Chapter 8

Clinical, cellular, and molecular aspects of arterial calcification

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Arterial calcification is a complex and independently regulated process with risk factors similar to those for atherosclerotic occlusive disease. It may develop either within the atherosclerotic intima or in the media. When calcification is found in coronary or lower extremity arteries, it is an independent predictor of cardiovascular events and lower extremity amputation. Recent evidence suggests a role for several endogenous stimulators and inhibitors in the pathogenesis of arterial calcification. Inflammatory mediators and matrix-degrading enzymes are also thought to control the progression of calcification in humans. Current research involves efforts to define the complex interactions between cellular and molecular mediators of arterial calcification. (J Vasc Surg 2007;45:57A-63A.)

Arterial calcification was previously viewed as an inevitable, passive, and degenerative process that occurred at the end stages of atherosclerosis. Recent studies, however, have demonstrated that calcification of arteries is a complex and regulated process.[1,2] It may occur in conjunction with atherosclerosis or in an isolated form that is commonly associated with diabetes and renal failure.[3,4] Higher artery calcium scores are associated with increased cardiovascular events, and some aspects of arterial calcification are similar to the biology of forming bone.[5,6] Arterial calcification can thus be viewed as a distinct inflammatory arteriopathy, much like atherosclerosis and aneurysms, with its own contribution to cardiovascular morbidity and mortality.[7]

Current research involves efforts to define the complex interactions between cellular and molecular mediators of arterial calcification and, in particular, the role of endogenous calcification inhibitors. This review discusses the clinical relevance, cellular events, and suspected molecular pathways that control arterial calcification and highlight some potential avenues for future research.

CLINICAL ASPECTS

Population studies. At least one third of Americans aged >45 years have arterial calcification when assessed by computed tomography (CT).[8,9] Risk factors for the development of early coronary artery calcification are similar to those for atherosclerotic occlusive disease and include hypertension, increased body mass index, and low high-density lipoprotein cholesterol.[10] In asymptomatic volunteers aged >50 years, coronary artery calcification is associated with increasing age, triglycerides, cholesterol, and increased intra-abdominal fat.[11]

The presence of diabetes significantly raises the risk of coronary calcification, and end-stage renal disease is thought to result in increased arterial calcium accumulation due to abnormalities in calcium and phosphorus homeostasis.[12-14] Common risk factors between atherosclerosis and calcification, and the high incidence of concurrent disease, have complicated our ability to separate the contribution of each to clinical events.

Recent data on lower extremity amputation, mortality, and coronary events confirm a strong predictive role of arterial calcium accumulation that persists even after correction for traditional risk factors. For example, when high coronary artery calcification is identified by electron beam or spiral CT, it predicts a fivefold to sevenfold increase in the risk of hard coronary events and this risk persists despite correction for age.[15,16] Similar predictive value is associated with calcification identified in the aortic arch.[17] When arterial calcification is specifically identified in the mid-thigh femoral artery, it is associated with a nearly threefold increase in the risk of amputation.[18,19]

Although the mechanisms whereby arterial calcification leads to increased vascular events are currently unknown, recent data suggest that coronary events and end-organ damage are related more to increased arterial stiffness rather than to the associated occlusive lesions.[20,21] Further attempts to characterize the effects of increased arterial stiffness on end organs are needed, as are methods to distinguish the effects of calcification from those of atherosclerotic lesions.

Histology. Vascular calcification has been divided into four forms that involve (1) the atherosclerotic intima, (2) the media of medium and large sized arteries, (3)

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This research was supported by a Mentored Clinical Scientist Development Award from the NIH HL069926, a grant from the NIDDK DK067368, and grants from the Lifeline Foundation, and the William J. von Liebig Foundation.
Competition of interest: none.
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0741-5214/$32.00

doi:10.1016/j.jvs.2007.02.049

cardiac valves, and (4) a widespread form known as calciphylaxis.[22] The second form, known as medial artery calcification or Mönckeberg sclerosis, is of primary importance in the diabetic population and particularly in patients with diabetic tibial artery disease. Medial artery calcification may be found in conjunction with atherosclerotic lesions, or it may occur independently.[2] It occurs in patients with normal serum calcium and phosphorus levels, and this distinguishes it from vascular calciphylaxis, which is related to high serum calcium and phosphate levels that result in passive mineral deposition onto elastic fibers.[22] Of these forms, medial artery calcification has been most strongly associated with cardiovascular events.[18]

The expression of bone-related genes in atherosclerotic lesions was described more than a decade ago.[23,24] Arteries from patients with medial calcification have increased expression of osteoprotegrin, a member of the tumor necrosis factor (TNF) ligand and receptor-signaling family. This is associated with apoptosis that occurs next to areas of recent calcification.[25] In clinical specimens, medial artery calcification occurred next to medial smooth muscle cells (SMCs) and in the absence of macrophages or lipids. Compared with control arteries, these vessels expressed less matrix Gla protein (MGP) and osteonectin and more alkaline phosphatase, bone sialoprotein, bone Gla protein, and collagen II, all indicators of osteogenesis or chondrogenesis.[26]

Cells residing in the media of human calcified arteries lose their expression of SMC markers and begin to express more osteogenic or chondrogenic markers, including the osteochondrogenic transcription factors Cbfa1, Msx2, and Sox9.[27] These studies on human specimens show arterial changes that resemble the biology of developing bone. They have helped us form hypotheses on the basic mechanisms that initiate and regulate arterial calcification.

Although our understanding of the specific pathologic events that incite arterial calcification remains limited, the factors that predispose to its development have recently been defined, and attempts to place these findings in perspective have begun.[2] It is thought that poor glucose control leads to a low-grade inflammatory response in the adventitia that is related to chronic metabolic, osmotic, and oxidative stresses.[28] These oxidative and hyperlipidemic signals may then induce the secretion of inflammatory mediators such as TNF-α, interleukin (IL) 6, and adipokines from macrophages in the arterial wall.[29,30] Moreover, patients with elevated levels of soluble IL-2 receptor, a marker of T-cell activation, have increased rates of coronary calcium progression compared with controls.[31] The complex interplay between risk factors, inflammatory mediators, and the development of occlusive vs calcific lesions will need further evaluation and scrutiny.

CELLULAR ASPECTS

The media: smooth muscle cells and calcifying vascular cells. The ability of medial SMCs to undergo phenotypic transformation was recognized >25 years ago by Campbell et al.[32] Phenotypic SMC changes that occur during calcification have only recently been described, however.[33] SMCs cultured from the aortic wall have multilineage potential with the ability to express chondrogenic, leiomyogenic, and stromogenic markers, but not adipogenic markers.[34]

Studies by Demer et al[35] have demonstrated that certain populations of medial SMCs, when grown in culture, are capable of undergoing calcification. These cells, known as calcifying vascular cells (CVCs), can differentiate into osteoblast-like cells while losing their ability to express smooth muscle-specific markers.[36] This suggests that the changes in cellular phenotype that occur during calcification may occur through the transformation of a subpopulation of medial SMCs into more osteogenic cells, and these changes may occur in the setting of diminished inhibitors or increased stimulators of calcification. The in vivo origin of these osteoblastic cells, however, has not been determined.

The adventitia: pericytes, fibroblasts, and mesenchymal stem cells. A program of osteogenic expression has been described in the arterial wall of *LDLR*$^{-/-}$ mice fed a high fat, diabetogenic diet.[28] This work, done by Towler et al,[37] demonstrated up-regulation of osteoblastic transcription factors Msx2 and Msx1 in perivascular adventitial cells. Msx2, which is thought to act in response to stimulation by bone morphogenetic proteins (BMPs), stimulates osteogenesis in adventitial myofibroblasts and in cells from a mesenchymal stem cell line while inhibiting adipogenic differentiation. In addition, mice carrying the CMV-Msx2 transgene have increased expression of Msx2 only in the adventitia, whereas alkaline phosphatase expression is increased only in the media.[38]

They further evaluated conditioned media from Msx2-stimulated mesenchymal cells and found that it induced expression of Wnt signaling molecules, TCF/Lef transcription, and nuclear β-catenin localization with concomitant alkaline phosphatase induction.[38] This strongly suggests that a subset of adventitial cells expressing a bone morphogenic protein 2 (BMP-2)-Msx2 signaling program can influence the phenotype of medial cells and may be responsible for inducing calcification.

In cell culture experiments on microvascular pericytes, dexamethasone, a steroid with recognized stimulatory effects on vascular calcification, down-regulates molecules thought to act as calcification inhibitors including MGP, osteopontin, and vascular calcification-associated factor (VCAF).[39] Advanced glycation end products increase calcification and osteoblastic differentiation of microvascular pericytes.[40] Therefore, cells expressing osteochondrogenic markers during calcification may represent medial cells responding to adventitial signals or precursor cells that are recruited into the media from surrounding tissues or from the blood stream.

Angiogenesis and the role of endothelial cells. A potential association between angiogenesis and the progression of arterial calcification has recently been proposed.[41] According to this hypothesis, osteogenic signals or osteogenic precursor cells may be conveyed into the arterial wall by collateral vessels that develop in response to

Table. Factors involved in arterial calcification

Stimulators	*Inhibitors*
Inorganic phosphate[48-50]	Pyrophosphate[61]
TGF-β1[51]	Statins[62]
25-hydroxycholesterol[51]	N-3 fatty acids[63]
Estrogen[35]	Tropoelastin[64,65]
cAMP[36]	BMP-7[66]
MAP kinase[52]	**Bisphosphonates**[67-69]
Acetylated LDL[53,54]	***Matrix Gla Protein**[70]
Homocysteine[37]	**Osteopontin**[71,72]
Glucose [55]	*Osteoprotegrin[73]
Endothelin-1[56]	***NPP-1 via Pip**[73]
Elastin degradation products[43]	***Ahsg (Fetuin-A)**[74]
Pit-1[57]	***Smad6**[75]
Leptin[58]	***Klotho**[76]
BMP2-Msx2-Wnt[38]	***Carbonic anhydrase-2**[77]
MMPs[59,60]	

TGF, Transforming growth factor; *cAMP,* cyclic adenosine monophosphate; *BMP,* bone morphogenic protein; *MAP,* mitogen-activated protein kinase; *LDL,* low-density lipoprotein; *NPP,* nucleotide pyrophosphatase phosphodiesterase; *PPi,* inorganic pyrophosphate.
Factors shown to affect calcification in vivo are in bold.
*Denotes data from mice with spontaneous or targeted gene alteration.

inflammatory signals. This theory is based on the known relationship between angiogenesis and the progression of atherosclerotic plaques.[6] Inhibitors of angiogenesis prevent the progression of atherosclerotic plaques,[42] whereas stimulators of angiogenesis enhance plaque progression.[43]

Inflammatory collaterals have been shown to originate from the adventitia.[44] Once established, it is proposed that these collateral vessels may allow for the migration of multipotent adventitial myofibroblasts or pericytes into the atherosclerotic intima or media where they can differentiate into osteoblast-like cells through the effects of bone-signaling proteins such as the BMPs and osteoprotegrin. Alternatively, the collaterals can be used to convey signals secreted by adventitial cells to the media, where they can affect the phenotype of calcifying vascular cells.

Of note is that arteries from rats that underwent removal of the adventitia had reduced amounts of medial calcification on a calcification-inducing diet.[45] In addition, endothelial cells exposed to atherogenic stimuli in culture demonstrated increased expression of MGP and BMP2.[46] Endothelial cells grown in culture with CVCs can either inhibit or stimulate calcification, depending on the specific culture conditions, and this is thought to be related to their expression of BMP2.[47] However, direct in vivo confirmation of a relationship between angiogenesis and arterial calcification has not been demonstrated.

MOLECULAR ASPECTS

Smooth muscle cell calcification in vitro. Numerous factors have been shown to affect the ability of media-derived cells to undergo calcification in vitro (Table).[48-77] Shortly after their identification of CVCs from bovine aorta, Demer et al[51] demonstrated that in vitro calcification was enhanced by the addition of two atherosclerosis-promoting proteins—transforming growth factor β1 (TGF-β1) and 25-hydroxycholesterol—and they suggested a link between calcification and atherosclerosis.

Other factors thought to be involved in atherosclerosis progression were also found to affect the ability of CVCs to calcify. For example, CVCs are responsive to the effects of estrogen[35] and to the effects of the intracellular-signaling molecules cyclic adenosine monophosphate[36] and mitogen-activated protein kinase (MAPK).[52] N-3 fatty acids inhibit calcification of CVCs though p38-MAPK and peroxisome proliferator-activated receptor-γ pathways,[63] and leptin, the circulating protein product of the *ob* gene, promotes osteoblastic differentiation and calcification of CVCs by increasing alkaline phosphatase activity.[58] In addition, macrophages are thought to exert their procalcification effects on CVCs through secretion of TNF-α.[29]

Cultured SMCs are also capable of undergoing calcification and expressing osteogenic phenotypes when they are exposed to high levels of inorganic phosphates.[78,79] This process is stimulated by high glucose, which increases the expression of Cbfa1 and BMP-2,[55] and it is inhibited by osteopontin and inorganic pyrophosphate.[48-50] Acetylated low-density lipoprotein and homocysteine stimulate vascular SMC calcification,[53,54] whereas statins prevent calcification through effects on the Gas6-Axl survival pathway.[62] Endothelin-1 blockade prevents calcification of cultured rat SMCs and it also has effects in vivo.[56] Matrix proteins, including elastin precursors, elastin degradation products, and decorin, a proteoglycan found in human calcified plaques, are also known to enhance SMC calcification in vitro.[65,65,80]

Of interest is that BMP-7 may counter the calcification-inducing effects of BMP-2 by promoting the SMC phenotype.[66] SMC calcification and phenotypic transformation into chondrocyte-like cells is also affected by altering levels of the sodium-dependent phosphate cotransporter Pit-1, suggesting that phosphate uptake by Pit-1 is essential for phosphate-induced SMC calcification and phenotype changes.[67]

Arterial calcification in rats: calcium, phosphorous, and bone resorption. Much of the early experimental data on medial calcification came from studies in rats overdosed with vitamin D.[81] Arterial calcification in this model is accompanied by calcification in the lungs and other tissues. It is most similar to human calciphylaxis.[2,82] High serum levels of calcium and phosphate are similar to levels seen in dialysis patients and those with parathyroid axis dysfunction.[22] Vitamin D_3 also increases vascular SMC calcification in vitro, and this effect is partially mediated by inhibition of parathyroid hormone–related peptide expression.[83]

The earliest finding in calcification induced by vitamin D_3 is that of matrix vesicles developing along elastin fibers, and this is similar to what has been seen in human specimens.[84,85] This is followed by deposition of calcium hydroxyapatite crystals along the elastic lamellae and degeneration of medial elastin.[84,86] Once the calcification process is initiated, it is thought to spread along the elastin fibers, ultimately leading to destruction of the elastin fibers them-

selves and then spreading to the surrounding medial smooth muscle cells.[59] Calcification in this model is generally thought to occur in the absence of an inflammatory cell infiltrate and is linked to bone resorption.[87]

Characterized by decreased levels of circulating fetuin-A, it can be delayed by inhibitors of bone resorption, including the cytokine osteoprotegrin and the amino bisphosphonates.[67-69] Calcification can also be reduced by V-H^+-adenosine triphosphatase inhibitor SB 242784[88] and sevelamer, a phosphate binder that does not contain calcium.[89] These studies by Price et al suggest that arterial calcification may be related to serum factors emanating from resorbing bone.

In humans, there is a known association between osteoporosis and arterial calcification.[90,91] Although calcification in these models is not thought to involve an inflammatory intermediate, it is likely that certain mechanisms overlap with those of other forms of calcification. For example, localized changes in extracellular calcium or phosphorus concentrations may be involved in the initial stages of medial and intimal calcification or in their progression.[33] In addition, we have recently shown that systemic administration of the antibiotic doxycycline, which has properties that inhibit matrix metalloproteinase activity, can reduce arterial calcification in rats treated with vitamin D.[60]

Another model of arterial calcification in rats involves the use of warfarin and vitamin K that are thought to work by reducing levels of MGP. Arterial calcification in warfarin-treated rats is accelerated by growth and vitamin D.[92] It can be reversed by treatment with an endothelin receptor antagonist and this appears to be dependent on activation of membrane bound carbonic anhydrase IV.[93] Finally, arterial calcification can also be induced in rats by feeding with an adenine-containing diet that causes uremia.[94] It has recently been demonstrated that calcification in this model is increased by diets low in protein and reduced by the bone resorption inhibitor ibandronate.[95]

Studies using rats injected with vitamin D have yielded significant information during the last 40 years; however, calcification in this model appears to be most similar to human vascular calciphylaxis because rats do not develop intimal lesions or similar inflammatory responses. Therefore, recent work in genetically altered mouse strains that spontaneously develop arterial calcification gives new opportunities to study this complex process.

Arterial calcification in mice: role of endogenous inhibitors. Evidence for endogenous calcification inhibitors comes from targeted or spontaneous gene alterations in mice. Mice deficient in the vitamin K–dependent protein MGP, a mineral-binding extracellular matrix protein, have chondrocytes within the arterial wall. They develop arterial calcification shortly after birth, and this results in death from arterial rupture in <3 months.[70] The mechanisms by which MGP deficiency leads to increased calcification remain unknown but may be related to interactions between MGP and BMP2.[96,97] Another potential interaction occurs between MGP and osteopontin, which also accumulates adjacent to calcifying areas. Mice deficient in both MGP and osteopontin had twice as much arterial calcification and died significantly earlier than control MGP-deficient mice.[70,98] Thyroid hormone is also thought to increase vascular SMC calcification through upregulation of MGP.[99]

Mice with a genetic deficiency leading to decreased inorganic pyrophosphate production develop spontaneous calcification through an endochondral process.[61] The decrease in inorganic pyrophosphate production is related to a mutation that inhibits the catalytic activity of nucleotide pyrophosphatase phosphodiesterase 1 (NPP-1). This mutation was initially identified as a cause of most of the cases of human idiopathic infantile arterial calcification.[100] Cultured aortic vascular SMCs isolated from mice deficient in NPP-1 had increased expression of the chondrogenic marker type II collagen and increased expression in aortic specimens of the mature chondrocyte marker type IX/XI collagen. In an organ culture model of aortic calcification, high phosphate and calcium levels are unable to induce calcification because of inhibition by pyrophosphate.[50]

Another genetic alteration resulting in spontaneous arterial calcification is a mutation in the gene encoding for osteoprotegrin, a member of the TNF superfamily of proteins, which is also expressed in human atherosclerotic lesions. It is a secreted osteoclast inhibitor, and mice deficient in osteoprotegrin demonstrate decreased bone density and increased aortic and renal artery medial calcification.[73] This appears similar to the known association between osteoporosis and arterial calcification in humans.[91] Apolipoprotein $E^{-/-}$ mice with inactivated osteoprotegrin demonstrate accelerated atherosclerotic lesion progression and calcification with reduced cellularity of lesions.[101] Osteoprotegrin also inhibits calcification in rats given warfarin or high doses of vitamin D.[82]

More recently, the serum protein α_2-Heremans-Schmid glycoprotein (Ahsg, also known as fetuin-A) has been identified as an important inhibitor of ectopic calcification. Mice deficient in Ahsg are phenotypically normal but develop calcification when placed on a diet containing increased calcium, phosphate, and vitamin D.[74] Arterial calcification occurred in the absence of elevated serum calcium or phosphorus levels and resulted in increased blood pressure and renal failure. In vitro experiments showed that lack of Ahsg was associated with a lack of inhibitory activity of serum proteins on bone calcium phosphate precipitation, suggesting that the effects of Ahsg were systemic rather than specifically on the arterial wall. This study supports the notion that many factors in addition to serum calcium and phosphorus levels are important in the development of arterial calcification, even in patients with renal failure.

Three additional mouse strains are known to develop calcification, but mechanistic studies have not been reported. Mice without the gene encoding for the Smad6 homologue Madh6, an intracellular inhibitor of TGF-β signaling, develop arterial calcification and cartilaginous metaplasia possibly through release of the BMP-2 signaling cascade.[75] Mutations in the mouse *klotho* gene cause a phenotype that resembles

human aging and medial calcification develops that may be related to increased serum phosphate levels.[76] Mice with a defect in the gene encoding for carbonic anhydrase 2 show calcification in small arteries.[77] Attempts to define the mechanisms by which these factors, and others, lead to arterial calcification are ongoing.

Matrix degradation in arterial calcification. Matrix metalloproteinases (MMPs) are known to play diverse roles in arterial pathologic processes.[102] They have been demonstrated to exert multiple effects during atherosclerosis, restenosis, and aneurysmal degeneration.[102] A role of MMPs in arterial calcification was previously demonstrated by Basalyga et al,[59] who focused on the importance of elastin degradation. Their work suggests that elastolytic MMPs are secreted by inflammatory cells in the adventitia or media, with consequent degradation of medial elastin. This leads to the release of elastin degradation products that, in turn, act as chemokines to recruit other inflammatory cells. A cycle of MMP-mediated elastin degradation, recruitment of inflammatory cells, and more MMP secretion ensues.

Inhibiting MMP activity would serve to stop this cycle and prevent the influx of new inflammatory cells. We have recently demonstrated efficacy of an MMP inhibitor in limiting calcium chloride–induced aortic calcification. We observed reduced aortic calcification in rats that were treated with GM6001, a nonselective, synthetic MMP inhibitor.[92]

MMPs are also known to have nonmatrix effects during osteogenesis,[103] and they are involved in several aspects of mesenchymal stem cell renewal and differentiation.[104] Recent work has demonstrated that osteopontin promotes pro-MMP9 activation in aortic mesenchymal cells.[105] This suggests that the role of matrix-degrading enzymes during arterial calcification may go beyond that of degrading medial elastin. Current work in our laboratory is focused on addressing these issues.

CONCLUSIONS

Artery calcification is a complex and regulated process that may occur in conjunction with atherosclerosis or in a more isolated form. It is now known to be an independent predictor of cardiovascular events, including lower extremity amputation. Multiple stimulators and inhibitors control the progression of vascular calcification, and it is likely to involve inflammatory mediators emanating from cells located in the arterial wall or the surrounding adventitia. Matrix effects also control the progression of calcification. Future work will involve unraveling of the molecular events leading to calcification and the development of novel molecular-based therapies to prevent its progression.

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Submitted Jan 19, 2007; accepted Feb 17, 2007.